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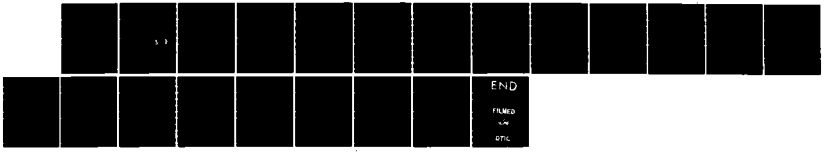

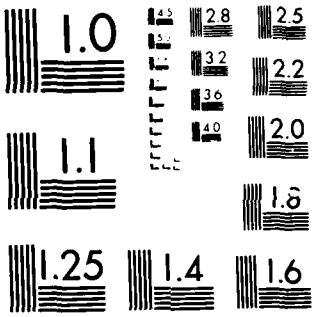

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Bioanalytical applications of fluorescence quenching are described. Both static and dynamic quenching processes, as applied to biochemistry, are reviewed. New developments and future trends of research in the area are discussed with regard to improved measurements.

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TECHNICAL REPORT NO. 8

Bioanalytical Applications of Fluorescence Quenching

by

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Prepared for Publication

in Trends in Analytical Chemistry

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Introduction

Luminescence measurements are important in theoretical studies as well as for diverse applications in other fields. There has been a steady proliferation of literature[1,2] on the subject over the past few decades. The popularity and applicability of the technique is largely attributed to its sensitivity and selectivity. The luminescence phenomena can be classified into two fundamental categories depending on whether the emission occurs between states of the same or different multiplicities.[3] Fluorescence, which has a much shorter lifetime, is of the former while phosphorescence, with a relatively long lifetime, is of the latter. The fluorescence lifetime of most fluorophores is in the range of 10^{-9} seconds (nanoseconds). Collisional interactions at concentrations of 10^{-3} to 10^{-4} M are often on this same time scale. Hence, collisional deactivation of the excited state (dynamic quenching) is often a competitive process at room temperature. Figure 1 provides a Jablonski representation of excitation and related de-excitation phenomena. However, a variety of processes which can lead to a diminution of fluorescence intensity should be noted. The two most common of these processes are collisional dynamic quenching involving interactions of quenchers with the excited fluorophore, and static quenching due to the formation of a complex between the quencher and the ground state fluorophore. The details of these processes and the associated mathematical relationships will be discussed in a later section of this manuscript.

Fluorescence quenching is not always detrimental to the measurement process since a variety of applications of the phenomenon has been reported. For example, fluorescence quenching is widely used as a probe in many bioanalytical applications. Since a wide variety of

substances can act as quenchers, the experimentalist has the option of selecting an appropriate quencher to probe the microenvironment of interest. Thus, one can frequently identify the desired fluorophore-quencher combination which provides useful information about a particular bioanalytical system. A quencher can often be very selective for a given fluorophore due to the quenching mechanism. Hence, structural information about the individual molecules in a macromolecular structure can be discerned. The importance of this structural information for the study of many macromolecules justifies the popularity of quenching studies in biochemical research. Finally, these experiments can be conducted with relative ease and without the need for complicated instrumentation.

Theory

Although detailed analysis of the quenching mechanism is beyond the scope of this manuscript, a short review of the two important quenching mechanisms, i.e., static and the dynamic, is necessary. Figure 2 shows possible dynamic and static quenching mechanisms. Our discussion in this manuscript will primarily emphasize fluorescence measurements although phosphorescence quenching has also been explored.[4,5]

A) Dynamic Quenching

Dynamic quenching of a fluorophore can be mathematically described using the Stern-Volmer relation, i.e.[1,6]

$$\frac{F_o}{F} = 1 + K_q \tau_o [Q] = 1 + K_d [Q] \qquad (1)$$

where F_o and F are the fluorescence intensities of the fluorophore in the absence and presence of the quencher, respectively. The parameter K_q is the bimolecular quenching constant and τ_o is the lifetime of the excited fluorophore in the absence of a quencher. The parameter K_d can

be defined as the Stern-Volmer constant. According to equation (1), quenching data can be presented as a linear plot of the concentration of the quencher versus the ratio of the unquenched fluorescence to the quenched fluorescence. The slope of the plot determines the K_d parameter.

B. Static Quenching

Although dynamic quenching always gives a linear Stern-Volmer plot, the observation of a linear Stern-Volmer relationship does not necessarily affirm the presence of a dynamic quenching mechanism. A similar linear relationship can be derived for a static quenching mechanism. In this case, the ratio of the fluorescence intensity can be calculated as

$$\frac{F_o}{F} = 1 + K_{eq}[Q] \qquad (2)$$

In the derivation of this equation, it is assumed that quenching occurs as a result of the formation of a non-fluorescent complex between the ground state fluorophore and the quencher. In numerous cases the formation of a dark complex can explain the static quenching process. An alternative explanation is that the fluorophore is not completely quenched. Under such conditions, the quantum yield of the fluorophore decreases. Assuming a change in the quantum yield of the fluorophore after ground state complex formation, a modified Stern-Volmer equation can be derived.(7)

$$\frac{F_o}{F} = \frac{1 + K_{eq}c}{e + K_{eq}cd} \qquad (3)$$

where d and e are factors for the quantum yield change of the complexed and free forms, respectively.

This modified Stern-Volmer (MSV) equation can be useful for the interpretation of several biochemical systems where the quenching of the fluorophore is not complete. It has already proved valuable for describing the interaction of different cyclodextrin systems with the polynuclear aromatic compound, pyrene.[7] There are other cases where the Stern-Volmer plot deviates from linearity, depending on the actual quenching mechanism. Detailed analysis of such is beyond the scope of this manuscript.

C. Combined Static and Dynamic Quenching

Observation of a pure dynamic or static quenching process can be obstructed by combined processes. In these cases, the fluorophore might be quenched both by collisions in the excited state and by complex formation in the ground state with the same quencher. The Stern-Volmer equation becomes a second order function[1] due to this combined process:

$$\frac{F_o}{F} = (1 + K_d[Q])\ (1 + K_s[Q]) \qquad (4)$$

For this combined interaction, a concave curvature is usually observed in the Stern-Volmer plot. However, it should be noted that this concave curvature is not absolute proof for the presence of the combined dynamic and static quenching, since the heterogeneous emission systems described later can result in a concave plot.

In another approach, the factor $e^{V(a)}$ is introduced into the Stern-Volmer equation[8] in order to describe the combined static and dynamic quenching modes, i.e.

$$\frac{F_o}{F} = (1 + K_d[Q])\ e^{V[Q]} \qquad (5)$$

where the constant V represents an active volume element surrounding the excited fluorophore. The presence of a high concentration of fluorophore, or other strongly absorbing species which absorb radiation at the absorption and/or emission frequencies, is a potential problem in fluorescence measurements. This excessive absorption of incident or emitted radiation attenuates the flux of luminescence progressively as the radiation traverses the optical path resulting in a concomitant decrease in emission. This phenomenon is called "inner filter effect" and should be distinguished from true quenching. If the inner filter effect is significant, the Stern-Volmer plot becomes truly exponential,[9]

$$\frac{F}{F_o} = 10^{-A} \qquad (6)$$

where A is the absorbance of the quencher (Figure 3).

D. Complex Systems

In the presence of homogeneous emission, the Stern-Volmer equation and its modified forms describe the quenching process within measurable error. However, in most biochemical systems, especially in the presence of protein, the fluorescence will be heterogeneous.[10] In the case of heterogeneous emission, the system has two or more types of emitting centers. For such multiple quenching systems, the appropriate form of the Stern-Volmer equation will be

$$\frac{F_o}{F} = \left(\sum_{i=1}^{n} \frac{f_i}{1 + K_i[Q]e^{V_i[Q]}} \right)^{-1} \qquad (7)$$

where f_i is the fractional contribution of component i to the total fluorescence at a given wavelength and the other parameters are as

defined previously. Obviously, if the difference between the quenching constants is not large, the equation will be reduced to an approximately linear expression (Stern-Volmer).

Bioanalytical Applications

One of the major areas of application of fluorescence quenching is in studies of proteins and other macro-biomolecules. This subject has been discussed recently in several excellent reviews.[6,11] Most of these studies use the native fluorescence of tryptophanyl residues and measures the effects of different quenchers on the fluorescence intensity of these residues. Extrinsic fluorescence via labels or probes, i.e. fluorescent molecules or other ligands which can be attached to specific sites on the biomolecules, are also being employed. This latter methodology expands the application horizon in addition to providing flexibility and selectivity in the measurement process.

Fluorescence quenching experiments are rather simple to perform, and an experienced researcher can obtain a wealth of information from such data. These include the extent of exposure of the tryptophanyl or the probed groups, the microenvironment in which these groups exist, and the overall conformation of the macromolecules and its dynamics, i.e., how the conformation may change with time. Such quenching experiments can be coupled with other analytical techniques to extend the dimensionality of the data, thus substantially increasing the information content.

The literature for the use of fluorescence quenching in biological studies is substantial, and we present here only a few representative examples of quenching applications. This brief review will hopefully

serve to show the diversity of areas, variety of quenchers, and versatility of the methods that can be used in the general fluorescence quenching scheme of analysis.

A number of quenchers have been tried and found useful for bioanalytical studies. Of the quenchers employed, iodide, oxygen and acrylamide are the three most common and successful. Of course, a large number of quenchers have yet to be examined in depth for bioanalytical applications.

The halogenated compounds have been used as quenchers for many years. For example, Lehrer[12] has studied in detail the applicability of iodide in the quenching of tryptophan fluorescence using model compounds and the protein lysozyme. He derived and applied a modified Stern-Volmer equation for the analysis and interpretation of fluorescence quenching data from proteins which contain more than one tryptophan side chain. Note in Figure 4 that the Stern-Volmer equation was modified such that a plot of $F_0/\Delta F$ provided a better explanation of the data.

Iodide is found to be an efficient dynamic quencher because of its charge and its size. In protein studies, iodide has been found to quench mainly the surface tryptophanyl fluorescence and denotes the degree of exposure of these groups in such systems. Iodide is also very sensitive to local charge densities. Thus, the nature of the microenvironment can be estimated since the presence or absence of charged or polar groups in the vicinity of the fluorophore can affect the local concentration of iodide and thus alter the quenching efficiency.

One can also estimate the viscosity of the microenvironment for dynamic quenchers since the viscosity of the solvent influences the rate of quenching by affecting the rate of diffusion of quenchers towards the fluorophore. Diffusion within the protein shows less dependence on the viscosity of the environment. Denatured protein is usually quenched by iodide with 100% efficiency. Thus, the quenching of protein fluorescence by iodide ion can indicate the degree of exposure and accessibility of the fluorescent residues and one can infer from these data some structural information.

Oxygen has also proved to be a useful quencher for studying protein systems. Weber et al.[13,14] have provided most of the interpretation and usefulness of fluorescence quenching studies by oxygen. Unlike the iodide ion, the oxygen molecule is small, neutral and apolar. It has been found to quench dynamically and efficiently with large quenching constant. Because oxygen is devoid of charge effects, its use eliminates uncertainties in cases where charge effects may play a role in the observation of fluorescence quenching. This aspect is contrasted with iodide quenching in the interpretation of quenching data by oxygen of ethidium bromide DNA complex.[13] This complex contains the positively charged eithinium bromide and negatively-charged phosphate groups which can affect the local concentration of charge sensitive quenchers due to attraction of opposite charges. By using oxygen as a quencher, Weber and coworkers have unambiguously concluded that the fluorophore is intercalated and protected by the helix and that oxygen must collide with the fluorophore for quenching to occur. Lakowicz and Weber[14] were also able to propose that proteins do not have rigid conformations. Rather, they undergo very rapid fluctuations on a nanosecond time scale. They arrived at this

conclusion because oxygen could very effectively quench tryptophanyl residues which were known to be buried deep into the known protein structures. Some of these conformations allow oxygen to diffuse into the protein.

The use of acrylamide as a quencher of indole fluorescence was investigated by Eftink and Ghiron.[8] They found that acrylamide is an efficient quencher and can quench via both a dynamic and static mechanism. Acrylamide is a neutral quencher like oxygen. However, it is larger, more polar and very soluble in aqueous solution. In their evaluation of acrylamide as a quencher, they used an indole-micelle complex to simulate a simple protein structure.[8] They were able to obtain information about the microenvironment of the fluorescence probe and the general structure of the complex. One other advantage of acrylamide over other neutral and nonpolar quenchers like oxygen and trichlorethanol is that it does not seem to interact with proteins to any significant extent. In another study of trichlorethanol as a quencher, Eftink *et al.*[15] found that trichlorethanol can localize in the apolar region of the macromolecule resulting in observable changes in quenching efficiencies.

Many other quenchers have been used for bioanalytical studies. In their review, Eftink and Ghiron gave a table summarizing quenchers which have been used to date.[6] Peterman and Laidler[16] have applied N-Bromosuccinamide quenching to study the reactivity of tryptophan residues in serum albumins and lysozyme via a kinetic method. Recently Ando and Miyata[17] found pyruvate ion to be a useful negatively charged quencher which is not toxic to protein. Moreover, pyruvate is present in many important biochemical reactions such that assays through coupled reactions involving pyruvate can be developed.

Fluorescence quenching has also been used to investigate the characteristics of biological membranes. In such studies, extrinsic fluorescence via a fluorescent probe is often employed. For example, Chaplin and Kleinfeld[18] used three different quenchers to study how amphipathic probes such as n-(9-anthroyloxy) fatty acids (AO) distribute themselves in biological membranes. The quenchers were neutral acrylamide, negatively charged iodide and positively charged copper (II) ions. They found that for vesicles of egg phosphatidylcholine, acrylamide did not quench the fluorescence of the probes associated with the vesicles except for the vesicle with the shortest chain. The quenching by iodide varied linearly with the position of the fluorescent moiety (AO) on the fatty acid chains. Copper (II) ions produced little or no quenching. The authors concluded that the probes were buried in the membrane (acrylamide is very water soluble and tends to stay in the aqueous layer) and that the probes were at different depths depending on the position of the AO moiety. Copper (II) ions did not penetrate much of the bilayer.

Fluorescence quenching has also been applied to the elucidation of the nature of binding sites between ligands with biomolecules. Winkler was one of the early workers in applying fluorescence quenching to such studies. He used bromate in quenching of aminonaphthalenesulfonic acids fluorescent probes to investigate the nature of binding sites of the probes with albumins and homologous antibody.[19] Recently, Toulme and coworkers[20] used the quenchers trichlorethanol to investigate the role of aromatic amino acids in the binding of gene 32 protein from bacteriophase T4 to single stranded nucleic acids. They found that the gene, in the presence of trichlorethanol, upon irradiation, yielded

only one type of photoproduct whose fluorescence was completely quenched. But actually, not all of the tryptophan residues were photochemically modified; instead, two of the five residues were protected. The new protein, because of its modified tryptophanyl residue did not bind well to DNA.

Chen has proposed an interesting method called "fluorescence quenching release" for the assay of enzymes.[21] In this method, he uses a concentrated fluorescent dye encapsulated in lecithin liposomes. In this manner the fluorescence is self-quenched. When the liposomes are disrupted, the dye is released and fluorescence is observed. Thus, the enzymes (in this case phosphorylase C) which can hydrolyze the lecithin, can be determined by measuring the released fluorescence. More elaborate applications of fluorescence quenching in studies of enzymes, their structure, nature of binding sites and activity can be found in the discussions of *Prasad et al.*[22] *and Sumegi et al.*[23]

In addition to simple quenching experiments, other parameters of fluorescence can be combined to add to the dimensionality of the data to obtain more specific information that simple quenching procedures cannot provide. For instance, measuring the anistropy of protein fluorescence in the presence of a quencher can rather conveniently provide information about the dynamics, e.g. rotation and segmental mobilities of the fluorescing residues or probes.[24] Recently, Maliwal and Lakowicz studied the effect of ligand binding and associated conformational changes in protein by using oxygen quenching and fluorescence depolarization of the tryptophan residues.[25] This type of technique will be applied more and more as the experimenters try to obtain better resolution of the problems to be solved.

New Developments and Future Trends

The increased applications of fluorescence quenching in biological studies is certain to continue. It seems very likely that multidimensional techniques will be employed to obtain desired information. For example, Pesek and coworkers[26] have recently combined fluorescence quenching and halide-ion nuclear magnetic resonance spectroscopy to study the binding of metals to proteins at low concentrations. Using the two techniques together, they could obtain information about the dynamics and exposure of the probes at the binding site.

Recent technological advances have allowed the development of a new generation of fluorimeters. Two video-fluorimeters with a multichannel imaging detector capable of simultaneous acquisition of data as a function of multiple excitation wavelengths and multiple emission wavelengths has been described.[27,28] The advantages of this multichannel instrumentation are that fluorescence wavelengths can be monitored simultaneously in quenching studies. The utility of this approach has been demonstrated.[9] The increase in the dimension of the data requires new strategies for data reduction. With respect to fluorescence quenching, a ratio deconvolution algorithm has been developed and can be used for qualitative identification.[29] Such techniques may be applied to fluorescence quenching of protein and to simultaneously detect several spectra arising from the simultaneous interactions of several different quenchers. This should be more advantageous than just observing the spectral shift or intensity decrease in the conventional emission spectrum.

Recently, a new instrument for fluorescence detection of circular dichroism (FDCD) has been described which incorporates a multichannel diode array detector.[30] Circular dichroism is a very useful technique for structural determination of chiral biomolecules. The development of new chiral quenchers[31,32] (Figure 5) which can stereoselectively quench biomolecules, coupled with this new instrumentation, should provide a new avenue for studying protein-quencher interactions.

It should be obvious from the preceding discussions that fluorescence quenching has been shown to be of great value for studies of macro-biomolecules. The use of such methodology will likely continue to grow because of the diversity of information which can be obtained using selective quenchers. In the future, we anticipate the coupling of this technique with other multidimensional fluorescence methods to increase the information content of the data and to help the analyst gain greater insights from their measurements.[30]

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Figure Captions

Figure 1 Jablonski energy representation of excitation and relaxation phenomena. Singlet (S) and triplet (T) electronic levels are shown as boldface lines; lighter lines represent the vibrational levels. Continuous vertical lines indicate absorption or emission transitions, dotted lines indicate vibrational relaxation and wavy lines indicate quenching processes. A = absorbance; F = fluorescence; P = phosphorescence; (R) ISC (reverse) intersystem crossing; VR/IC = vibrational relaxation/interval conversion; Q/q = fluorescence/phosphorescence quenching.

Figure 2 Schematic representation of dynamic and static quenching mechanisms.

1. Excitation process followed by fluorescence.

2. Dynamic quenching of the excited (F*) fluorophore by a Q_d quencher.

3a. Static quenching via formation of a dark complex.

3b. Formation of a decreased quantum yield static complex.

Figure 3 Stern-Volmer plots for carbazole (+) and 9,10-dimethylanthracene (X) using nitromethane as a quencher. Carbazole shows the exponential behavior predicted by Eq. (6) indicating an inner-filter effect contribution, whereas 9,10-dimethylanthracene, anthracene, fluoranthene, and perylene all gave the expected linear plot. Reprinted from reference 9.

Figure 4 Modified Stern-Volmer plot of the quenching of lysozyme fluorescence by iodide. Reprinted from reference 12.

Figure 5 Stern-Volmer plots for quenching of fluorescence of the LADH-ϵNAD^+-pyrazole complex by L-, D-, and DL-methionine. The ternary complex was prepared in 0.05 M phosphate buffer (pH 7.3) and contained 1.2×10^{-5} M of both LADH and ϵNAD^+ and 1.7 mM pyrazole. (O) Quenching by L- or D-methionine. (●) Quenching by DL-methionine. (Δ) F_o/F values obtained by averaging the corresponding values of D- and L-methionine. Reprinted from reference 32.

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